LRP2 Acts as SHH Clearance Receptor to Protect the Retinal Margin from Mitogenic Stimuli

Highlights

- LRP2 modulates the SHH pathway in a context-dependent manner
- LRP2 clears and degrades SHH ligand from the murine retinal margin
- LRP2 activity keeps the ciliary marginal zone in a quiescent state
- Margin excess proliferation may underlie buphthalmos in LRP2-deficient patients

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In Brief
Christ et al. show that LRP2 mediates endocytic clearance of SHH in the murine retinal margin rather than acting as an auxiliary receptor. This context-specific function protects retinal progenitor cells from mitogenic stimuli and keeps them quiescent. Loss of LRP2 function leads to ectopic proliferation in the progenitor niche.
LRP2 Acts as SHH Clearance Receptor to Protect the Retinal Margin from Mitogenic Stimuli

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SUMMARY

During forebrain development, LRP2 promotes morphogen signaling as an auxiliary SHH receptor. However, in the developing retina, LRP2 assumes the opposing function, mediating endocytic clearance of SHH and antagonizing morphogen action. LRP2-mediated clearance prevents spread of SHH activity from the central retina into the retinal margin to protect quiescent progenitor cells in this niche from mitogenic stimuli. Loss of LRP2 in mice increases the sensitivity of the retinal margin for SHH, causing expansion of the retinal progenitor cell pool and hyperproliferation of this tissue. Our findings document the ability of LRP2 to act, in a context-dependent manner, as activator or inhibitor of the SHH pathway. Our current findings uncovered LRP2 activity as the molecular mechanism imposing quiescence of the retinal margin in the mammalian eye and suggest SHH-induced proliferation of the retinal margin as cause of the large eye phenotype observed in mouse models and patients with LRP2 defects.

INTRODUCTION

The low-density lipoprotein receptor-related protein (LRP) 2 is a member of the LDL receptor gene family, a group of structurally related cell surface receptors with important roles in embryonic development (reviewed in Willnow et al., 2012). Earlier, we uncovered the function of LRP2 as an auxiliary sonic hedgehog (SHH) receptor required for mammalian forebrain formation (Christ et al., 2012). At neurulation, LRP2 is expressed in neuroepithelial cells where it forms a complex with Patched (PTCH) 1 enabling SHH binding and signal reception in the rostral diencephalic ventral midline (RDVM), the major forebrain organizer region. Loss of LRP2 activity in mouse models results in the inability of SHH to induce its target field in the ventral midline, and, consequently, in midline formation defects and in holoprosencephaly (Christ et al., 2012). Holoprosencephalic features are also seen in patients with Donnai-Barrow syndrome, an autosomal recessive disorder caused by LRP2 defects, documenting the conservation of receptor function across species (Kantarci et al., 2007; Rosenfeld et al., 2010).

To explore the significance of LRP2 for SHH activity in paradigms other than forebrain patterning, we focused on retina development, a process dependent on SHH signals (Neumann and Nusslein-Volhard, 2000; Wang et al., 2002). Our choice was guided by large eye phenotypes seen in LRP2-deficient humans (Pober et al., 2009) and mouse and zebrafish models (Gajera et al., 2010; Veth et al., 2011). Yet, the mechanism of LRP2 action during eye development and whether it functions as SHH binding protein in this context remained unclear.

Here, we show that LRP2 is expressed in the peripheral margin of the murine embryonic retina where it modulates SHH signaling during retinal differentiation. However, in contrast to the situation in the RDVM, in the retinal margin, LRP2 acts as an inhibitory receptor that mediates cellular clearance of SHH, protecting the retinal margin from detrimental mitogenic stimuli by this morphogen. Absence of LRP2 in gene targeted mice results in ectopic induction of SHH signaling and aberrant expansion of the progenitor cell pool in the margin of the retina and in hyperplasia of peripheral retina tissue. These studies identified a clearance function of LRP2 as a mechanism to control SHH signaling in vivo and suggest excessive proliferation of the retinal margin as cause of the large eye phenotype in LRP2-deficient patients and mouse models.

RESULTS

Hereditary Buphthalmos in LRP2-Deficient Mice

We explored a role for LRP2 in the functional integrity of the mammalian eye, a process disturbed by receptor deficiency in humans and mice (Gajera et al., 2010; Pober et al., 2009; Storm et al., 2014). We carried out our analyses using two different Lrp2 null alleles that disrupt LRP2 synthesis in mice. Analyses of embryonic stages were performed in mice with targeted Lrp2 gene disruption (Lrp2<sup>−/−</sup>) on a C57BL/6N background (Spoelgen et al., 2005). For analysis of postnatal and adult stages, Lrp2<sup>−/−</sup> animals were crossed with the ENU line 267 that carries a premature stop codon in the sequence encoding the extracellular domain of LRP2 (Lrp2<sup>267/+</sup>) (Zarbalis et al., 2004). Compound heterozygous mice (Lrp2<sup>267/−</sup>) exhibit a higher rate of perinatal survival compared to Lrp2<sup>−/−</sup> animals due to the (FVB/NJ × C57BL/6J) background of line 267 (Gajera et al.,...
For both alleles, LRP2-deficient animals (Lrp2<sup>−/−</sup> or Lrp2<sup>+/−</sup>) were compared to their respective heterozygous or wild-type littermates (jointly referred to as controls).

As reported previously, adult LR2-deficient mice are characterized by craniofacial malformations, including a shortened snout and an open frontal fontanel, indicative of mild holoprosencephaly (Figures 1A–1D). However, adult Lrp2 mutant mice also exhibit enlarged and exophthalmic eyes (buphthalmos) (Figure 1E), a defect not typically seen in holoprosencephalic syndrome. Enlarged globes are already apparent at postnatal day (P) 10 (Figure 1F), with a 17% increase in axial length (Figure 1G).

The diagnosis of holoprosencephaly (Figures 1A–1D) was characterized by craniofacial malformations, including a shortened snout and an open frontal fontanel, indicative of mild holoprosencephaly (Figures 1A–1D). However, adult Lrp2 mutant mice also exhibit enlarged and exophthalmic eyes (buphthalmos) (Figure 1E), a defect not typically seen in holoprosencephalic syndrome. Enlarged globes are already apparent at postnatal day (P) 10 (Figure 1F), with a 17% increase in axial length (Figure 1G).

LRP2 Deficiency Causes Ectopic Induction of SHH Signals in the Retinal Margin

Because CB hyperplasia was seen at early postnatal stages in LR2-deficient mice, we suspected a developmental defect as cause of this phenotype. Thus, we characterized the expression of LR2 in wild-type embryos. Around E10.5, invagination of the neural plate was restricted to the peripheral margin as retina differentiation proceeds. At E16.5, LR2 is largely confined to the ciliary marginal zone (CMZ), which will give rise to the CB and iris in the postnatal eye (Figures S2B and S2C). In the CMZ, LR2 is positioned at the apical surface of non-pigmented ciliary epithelial cells (Figures S2B–S2D).

In the mammalian eye, SHH is produced by retinal ganglion cells (RGCs), resulting in patterning of the neural retina in a central to peripheral direction (Neumann and Nusslein-Volhard, 2000; Wang et al., 2002). SHH activity is absent from the CMZ that, in turn, receives signals from WNT ligands and bone morphogenetic proteins (BMPs) (Chao and Cepko, 2006; Zhao et al., 2002). To test whether loss of LR2 impairs SHH-dependent patterning of the neural retina, we analyzed markers of retinal differentiation. The histological appearance of the neural retina was comparable in control and Lrp2<sup>−/−</sup> eyes at E16.5 (Figures 2A and 2B). Also, expression of Shh in the retinal ganglion cell layer (Figures 2C–2F) and of the neurogenic marker Math5 (Figures 2G–2J) was normal. Overall correct patterning of the mutant neural retina was confirmed by immunodetection of Zic2 and Isl2, markers of postmitotic retinal cell types (Figures 2K–2P). However, a subtle, but consistent, alteration was seen for the retinal ganglion cell marker Bmi-3b. Expression of this marker was pronounced in the central retina in both genotypes, but seemed to extend into more distal regions of the retina in mutants (Figures 2R and 2S). Although the neurogenic marker Math5 was not induced in the mutant CMZ, the findings on Bmi-3b suggested that this niche might adopt a partial neurogenic character when LR2 activity is lost. To further substantiate changes in Bmi-3b expression, we dissected the neural retina and CMZ from E16.5 eyes for quantitative (q) RT-PCR. Transcripts of Bmi-3b and Lrp2 were prominent in the neural retina and CMZ, respectively, documenting proper tissue dissection (Figure S2E). In line with the observations made by immunodetection, transcript levels of Bmi-3b, but not of Zic2 and Isl2, were increased in the LRP2-deficient CMZ (Figure S2E).

Our data suggested a distal shift of the boundary between the central retina and CMZ in LR2-deficient eyes. A role for LR2 in specification of the retinal margin was confirmed by investigating morphogen pathways in the CMZ by in situ hybridizations (ISH). At E16.5, CMZ markers Bmp4, Msx1, and Otx1 were expressed at the correct position in mutant eyes, indicating proper establishment of the CMZ at the retinal periphery (Figure 3A). However, the SHH targets Hes1 and Gli1, which were not expressed in the CMZ of control eyes (Figures 3B, b, f, and j), were ectopically induced in the Lrp2<sup>−/−</sup> CMZ (Figures 3B, d, h, and j). Also, Ptc1 levels were increased (Figure 3C). Significantly elevated transcript levels for Gli1 and Ptc1 in the mutant CMZ, but not in the neural retina, were confirmed by qPCR (Figure 3D). Transcript levels of Shh were not changed in the neural retina or CMZ of the mutants (Figure 3D), in line with findings obtained byISH in Figures 2E and 2F above.

Aberrant induction of the SHH pathway in the mutant CMZ was confirmed by crossing the Lrp2 null allele with the Gli1<sub>LacZ</sub> reporter line (Bai et al., 2002). In E16.5 control eyes, Gli1-dependent lacZ activity was restricted to the neural retina, but absent from the CMZ (Figures 3E, a and b). In contrast, in Lrp2<sup>−/−</sup> eyes, lacZ activity was ectopically induced in the CMZ (Figures 3E, c and d). Induction of the SHH pathway in the Lrp2<sup>−/−</sup> CMZ coincided with loss of WNT activity in this tissue as shown by crossing the Lrp2 null allele with the Tcf/Lef<sub>LacZ</sub> reporter strain (Mohamed et al., 2004). As seen in Figure 3E, expression (Figures 3E, i–j) and activity (Figures 3E, e and f) of lacZ was seen in the CMZ, but not in the neural retina, of E16.5 control mice. In contrast, Tcf/Lef-dependent expression (Figures 3E, k and l) and activity (Figures 3E, g and h) of lacZ was significantly reduced in the Lrp2<sup>−/−</sup> CMZ. Loss of WNT activity in the E16.5 mutant eye was not due to earlier defects, as components of the WNT pathway, such as Wnt7b and G protein coupled receptor 177, were normally expressed in the mutant eye at E10.5–11.5 (Figure S3A).

Eye development not only depends on SHH, but also on the related morphogen indian hedgehog (IHH) produced by the choroid adjacent to the RPE (Dakubo et al., 2008). Expression of Ihh in the choroid was unchanged in Lrp2<sup>−/−</sup> mice (Figure S3B). Also expression of Pitx2, an IHH target in the choroid
Figure 1. LRP2 Deficiency Causes Hereditary Buphthalmos

(A–D) Adult Lrp2+/− mouse and littermate control. Note the exophthalmic eyes (buphthalmos) in the Lrp2+/− animal (C and D).

(E and F) Eye size at 10 weeks of age (E) and postnatal day (P) 10 (F). The scale bar represents 1 mm.

(G and H) (G) Axial length and lens thickness as determined on transverse sections of P10 eyes (exemplified in H, c). The axial length is increased in Lrp2+/− compared to control animals (n = 5 per genotype; **p < 0.01; and Mann-Whitney U test). The values are mean ± SEM (H). The H&E-stained transverse sections of adult and P10 eyes showing increased globe diameter (a–d), thinning of the retina (e–h), as well as hyperplasia of the non-pigmented epithelium of the CB (arrows; i–l) in Lrp2+/− animals in both age groups. The lines in (c) indicate the measurement of the axial length and lens thickness as quantified in (G).

(I) Analyses as in (H) for P0.5 eyes. The Lrp2−/− eyes show normal diameter (a and b) and retinal thickness (c and d), but hyperplasia of the CB (e and f). The immunohistological detection of LRP2 in the CB of all age groups is shown in (H, m–p) and (I, g and h). The scale bars represent 50 μm. See also Figure S1.
was not altered (Figure S3B), suggesting changes in the activity of SHH (not of IHH) as the cause for induction of the hedgehog pathway in the LRP2-deficient CMZ.

BOC, CDON, and GAS1 are also expressed in the CMZ, implicating several SHH binding proteins in formation of the retinal margin. Yet, the expression patterns for Boc, CDon, and Gas1 were indistinguishable comparing LRP2-deficient and control E16.5 eyes by ISH (Figure S3C) and qPCR (Figure S3D), excluding defects in expression of these proteins in the pathology of Lrp2−/− eyes.

Expansion of the RPC Pool in the LRP2-Deficient Retinal Margin

In the rodent retina, SHH acts as a potent mitogen that promotes RPC proliferation (Jensen and Wallace, 1997; Levine et al., 1997; Wang et al., 2002). The absence of Hh activity from the CMZ contributes to the quiescence of RPC in this niche in the mammalian eye (Moshiri and Reh, 2004). In line with this assumption, aberrant spread of SHH activity into the CMZ of the Lrp2−/− retina resulted in the expansion of the RPC pool in the retinal margin as documented by detection of progenitor cell markers SOX2 and VSX2 by immunohistology and qPCR. In wild-types, the expression domains for both progenitor cell markers sharply decreased distal to the boundary between the neural retina and CMZ (Figure 4A). In mutants, the signals were prominently induced in the CMZ (Figure 4A). Elevated levels of SOX2 and VSX2 in the CMZ (but not in the neural retina) were confirmed by quantification of immunosignals (Figure 4B) and by qPCR (Figure 4C).

In addition to increased RPC numbers, the mutant CMZ also showed enhanced proliferative capacity as verified by the quantification of cells in S phase using in utero labeling with BrdU.
LRP2 Deficiency Enhances SHH-Dependent Cell Proliferation in the CMZ

We applied a retinal explant model to dissect the pathways underlying the enhanced mitogenic potential of the LRP2-deficient CMZ at E16.5 (Figures S5A and S5B) (Liu et al., 2007). Retinal explants were cultured for up to 48 hr, retaining their identity as judged by expression of LRP2 and Msx1 in the CMZ, and of Bmp-3b in the neural retina (Figure S5C).

Initially, we investigated whether the LRP2-deficient CMZ in retinal explants showed higher proliferative capacity as seen in vivo. Applying BrdU labeling in explants cultured for 24 hr, we confirmed an increase in BrdU+ cells in the mutant compared to the control CMZ (Figures 5A and 5B). Enhanced proliferation in the mutant CMZ was dependent on SMO activity, as application of the inhibitor KAAD-cyclopamine decreased the number of BrdU+ cells in mutants to wild-type levels (Figure 5E). Application of JW55 (Waaler et al., 2012) (Figure 5E). Application of JW55 caused loss of WNT activity in the CMZ of control explants from wild-type mice (Figure 5D), arguing that the reduction of WNT activity in the LRP2-deficient CMZ, the activity of the GLI1-dependent LacZ reporter is aberrantly induced (d) as compared to the control tissue (b) (seen in nine out of 16 embryos). The activity of the Tcf/Lef-dependent lacZ reporter gene is diminished in the mutant (h) compared to the control (f) CMZ (in seven out of nine embryos). The reduction of Tcf/Lef-dependent lacZ expression in Lrp2−/− (l) compared to the control CMZ (j) was confirmed by ISH for lacZ (l-h). The ectopic induction was seen in three out of five (Hes1) and in four out of five (GlI1) embryos analyzed.

LRP2 Mediates Clearance of SHH to Antagonize Morphogen Activity

Having established an explant model that faithfully recapitulates features of the LRP2-deficient CMZ in vivo, we tested the
expression of SHH pathway components in this tissue in the presence and absence of LRP2. Primary cilia were abundantly present in the neural retina and, to a lesser extent, in the CMZ as shown by staining for the ciliary marker Arl13b (Figure 6A). In the control CMZ, LRP2 localized to the base of the primary cilium (Figure 6A, inset in d). No difference in the number of Arl13b+ cilia in the CMZ was seen comparing genotypes (Figure 6C). However, a striking difference was noted when cilia were costained for GLI2 and SMO as an indicator of pathway activation (Figure 6B). In controls, SMO signals were observed in GLI2+ primary cilia in the neural retina (Figure 6B, a), but were absent from cilia in the CMZ (Figure 6B, b). In contrast, robust ciliary staining for SMO was seen in the mutant CMZ (Figure 6B, d). An approximate 3-fold increase in SMO+ cilia in the CMZ was confirmed by quantification (Figure 6C), documenting aberrant activation of the SHH pathway in the LRP2-deficient retinal margin. The extent of SHH pathway activation matched well with a similar 3-fold increase in mitogenic stimuli received by the Lrp2-deficient CMZ (as shown by quantification of BrdU+, Ki67+, and PH3+ cells; Figures 4D–4G). As well as in retinal explants, this increase in SMO+ primary cilia in the mutant CMZ was also confirmed on histological sections of the E16.5 mouse eye (Figures S6A and S6B). Aberrant pathway activation in the CMZ coincided with a clear change in the distribution of endogenous SHH ligand in the mutant eye as shown by western blot analysis of dissected tissues. Although the levels of SHH protein were slightly lower in the neural retina, they were almost 2-fold increased in the CMZ of Lrp2−/− compared to control eyes (Figure 6D).

To explore the reason for accumulation of endogenous SHH in the mutant CMZ, we investigated the fate of the morphogen in the retina. When we incubated control explants with a...
recombinant fusion protein of SHH-N and GST, intracellular accumulation of the ligand was detected in the CMZ, but not in the neural retina (that does not express LRP2) (Figures 6E, a and b and e and f). Uptake was mediated by LRP2 as no uptake of GST-SHH-N was seen in the LRP2-deficient CMZ (Figures 6E, c and d). No internalization was observed for GST only (Figure 6E, a and b and e and f). No internalization was observed for GST only (Figures 6C). When targeted for endocytic markers, LRP2 localized to clathrin-coated pits (AP2), early endosomes (Rab4), and recycling endosomes (Rab11), but not to lysosomes (Lamp1) in the control CMZ (Figure 6F, upper). This pattern is consistent with the recycling fate of an endocytic receptor. In contrast, internalized GST-SHH-N localized to early endosomes (Rab4) and lysosomes (Lamp1 and cathepsin B), but not to recycling endosomes (Rab11) (Figure 6F, lower). This subcellular distribution of GST-SHH-N was surprising because in the RDVM, LRP2 delivers SHH to the Rab11+ recycling compartment, but not to lysosomes (Christ et al., 2012).

To substantiate lysosomal targeting of SHH in the CMZ, we performed uptake assays in control explants. First, we tested intracellular localization of GST-SHH-N in explants treated with chloroquine, an inhibitor of lysosomal degradation that causes swelling of the lysosomes. Application of chloroquine resulted in accumulation of large vesicles in the CMZ that costained for LRP2 and GST-SHH-N. This pattern was distinctly different from the apical vesicular signals seen for LRP2 and GST-SHH-N in untreated tissue (Figure S6D). Second, we coincubated control explants with GST-SHH-N and lactoglobulin, a ligand delivered to lysosomes. Both proteins colocalized in intracellular vesicles in the control CMZ (Figure S6E).

Conceivably, LRP2-mediated lysosomal catabolism of SHH antagonizes morphogen activity in target cells. This hypothesis was tested by luciferase reporter assay. To do so, conditioned media from HEK293 cells secreting SHH-Np were incubated at 37°C for 14–16 hr in empty 24-well plates or in 24-well plates containing a monolayer of rat choriocarcinoma (BN16) cells. BN16 cells express LRP2 and are commonly used to test the endocytic activity of this receptor. Western blot analysis confirmed depletion of SHH-Np from the supernatant of BN16 cells compared to buffer-treated tissues (seen in three out of six explants analyzed). (A) Immunodetection of BrdU+ cells in the CMZ of E16.5 control and Lrp2−/− retinal explants cultured for 24 hr. The CMZ is encircled by a white line. The BrdU+ cells in the neural retina (arrowhead) and lens (asterisks) are indicated. (B and C) Quantification of BrdU+ cells in the CMZ of control and Lrp2−/− explants after 24 hr incubation. The explants in (B) were incubated in 3 μM KAAD-cyclopamine or in solvent DMSO only. The explants in (C) were incubated in 800 nM SAG or buffer (n = 5–12 per genotype) (not significant: n.s.; **p < 0.01; ***p < 0.001; and two-way ANOVA followed by Bonferroni post test). The values are mean ± SD.

(D) Loss of lacZ activity in the CMZ (arrowheads) of control explants of the Tcf/Lef-lacZ line treated for 36–48 hr with 800 nM SAG as compared to buffer-treated tissues (seen in three out of six explants analyzed). (E) Wild-type retinal explants of the Tcf/Lef-lacZ line were treated for 24 hr with buffer (a and b), 5 μM JW55 (c and d), or 5 μM JW55 and 800 nM SAG (e and f). The suppression of WNT signaling in JW55-treated explants (reduced lacZ signal in c) does not increase cell proliferation in the CMZ (encircled) above levels in the buffer control as shown by BrdU staining (b and d; seen in eight out of eight explants). However, induction of proliferation in the CMZ is seen in JW55-treated explants that received SAG (f; seen in three out of three explants analyzed) (lens: le). The scale bar represents 75 μm. See also Figure S5.
BN16- and empty-well incubations was seen in the presence of anti-LRP2 antiserum (Figure S6).

**LRP2 Increases the Threshold for SHH Signals to Suppress Mitogenic Stimuli in the CMZ**

Data in Figures 6 and S6 provided cumulative evidence that LRP2 facilitates clearance of SHH by cells in the CMZ, thereby antagonizing morphogen activity in the retinal margin. To put this concept to the test, we compared the response of control and LRP2-deficient CMZ explants to SHH-Np (Figure S7A). Incorporation of BrdU served as a measure of the mitogenic potential of SHH in these tissues (Figure S7B). To do so, we used 48 hr incubations of retinal explants contrary to the analyses of endogenous SHH activity in experiments after 24 hr (Figures 5A–5C). In explants treated with blank medium for 48 hr, the proliferative capacity was low, reaching comparable levels in control and mutant CMZ. This residual proliferation was not inhibited by KAAD-cyclopamine (Figure 7C; condition 0 μg/ml SHH-Np). This finding is in line with earlier observations that loss of retinal ganglion cells depletes the source for SHH from retinal explants incubated for an extended period of time (Wang et al., 2002). Still after 48 hr, explants of both genotypes responded to exogenous SHH-Np from conditioned media by induction of proliferation in the CMZ as shown by BrdU incorporation. Proliferation was blocked by KAAD-cyclopamine (Figures 7A and 7B). Foremost, these data confirmed the ability of both genotypes to activate the ligand-dependent SHH pathway in the CMZ.

![Figure 6](https://example.com/figure6.png)

**Figure 6. LRP2 Mediates Lysosomal Catabolism of SHH to Antagonize Morphogen Activity**

(A) Immunodetection of Arl13b in CMZ and neural retina of Lrp2<sup>−/−</sup> and control E16.5 retinal explants. The Arl13b primary cilia (red) are seen in the neural retina and to a lesser extent in the CMZ (indicated by white lines) in both genotypes as shown in overview (a and b) and in higher magnification micrographs (c–f). The asterisks in (a) and (b) mark primary cilia in the lens. The insets show localization of LRP2 (green) at the ciliary base in control (d), but not Lrp2<sup>−/−</sup> (f) CMZ tissue.

(B) Immunodetection of SMO (red) and ciliary marker GLI2 (green) identifies cilia positive for SMO (arrowheads) in the mutant (d), but not in the control (b) CMZ. In the neural retina, SMO+ cilia are seen in both genotypes (a and c).

(C) Quantification of primary cilia positive for Arl13b and SMO in control and Lrp2<sup>−/−</sup> CMZ explants (as exemplified in A and B). The cilia per histological section was determined in n = 11–13 animals. (not significant: n.s.; **p < 0.001; and Mann-Whitney U test). The values are mean ± SD.

(D) Levels of endogenous SHH are slightly decreased in the neural retina, but increased in the CMZ of Lrp2<sup>−/−</sup> compared to control eyes as documented by western blotting of dissected tissues (exemplified in the inset) and by densitometric scanning of replicate blots (n = 4–7 pools per genotype; eight eyes per pool for the CMZ, two to five eyes per pool for the neural retina). The SHH levels in the mutant tissues were corrected for the tubulin loading control and are given as % signal of the respective control pool (set to 100%; column statistics and one sample t test).

(E) Immunodetection of LRP2 (green) and SHH (red) in control and Lrp2<sup>−/−</sup> retinal explants treated with GST-SHH-N. The overviews (a, c, e, and g) as well as higher magnifications (b, d, f, and h) of the neural retina and CMZ are shown. In controls, robust uptake of GST-SHH-N is seen in the CMZ (a and b), but not in the neural retina (e and f). No uptake of GST-SHH-N into the CMZ (c and d) or the neural retina (g and h) is seen in the Lrp2<sup>−/−</sup> explants.

(F) Control explants were incubated with 5 μg/ml GST-SHH-N and subsequently stained for LRP2 (upper) or GST-SHH (lower) and the indicated markers. LRP2 (green) localizes with markers (red) of clathrin-coated pits (AP2), early (Rab4), and recycling (Rab11) endosomes, but not lysosomes (Lamp1). In contrast, internalized GST-SHH-N (red) colocalizes with markers (green) of early endosomes (Rab4) and lysosomes (Lamp1 or cathepsin B [CatB]), but not with recycling endosomal marker Rab11. The scale bars represent 5 μm (A, c; B, a) and 25 μm. See also Figure S6.
An intriguing difference between control and \textit{Lrp2}^{-/-} explants was noted when their response to defined concentrations of exogenous SHH-Np was scored. At 0.05 \( \mu \text{g/ml} \) of SHH-Np, both genotypes reacted with an increase in BrdU+ cells in the CMZ as compared to blank medium or KAAD-cyclopamine treated tissues, but this increase was significantly higher in \textit{Lrp2}^{-/-} as compared to control explants (Figure 7C). This difference was not seen using 4-fold higher concentrations of SHH-Np, when both genotypes responded equally (Figure 7C; 0.2 \( \mu \text{g/ml} \)). To provide a quantitative description of the sensitivity

Figure 7. LRP2 Increases the Threshold for SHH Signaling to Suppress Mitogenic Stimuli in the Peripheral Retina

(A and B) Immunodetection of BrdU+ cells (red) in the CMZ of control and \textit{Lrp2}^{-/-} retinal explants cultured for 48 hr in the presence of 0.05 \( \mu \text{g/ml} \) (A) or 0.2 \( \mu \text{g/ml} \) (B) SHH-Np. Where indicated, the medium also included 1 \( \mu \text{M} \) KAAD-cyclopamine or the solvent DMSO. The CMZ is encircled by a white line. The scale bar represents 75 \( \mu \text{m} \).

(C) Quantification of the number of BrdU+ cells per CMZ section (as in A and B) for retinal explants incubated for 48 hr in the absence (0 \( \mu \text{g/ml} \)) or in the presence of the indicated concentrations of SHH-Np (as exemplified in A and B). The SHH-Np-induced stimulation of proliferation is blocked by 1 \( \mu \text{M} \) KAAD-cyclopamine in both genotypes (n = 3–14 per genotype) (*p < 0.05 and two-way ANOVA followed by Bonferroni post test).

(D) Dose-response curve of the number of BrdU+ cells in control and \textit{Lrp2}^{-/-} CMZ explants treated with the indicated concentrations of SHH-Np for 48 hr. The curves were fitted assuming a sigmoidal dose-response (variable slope). The comparison of fits: F (5.64; 1.54). The concentration of SHH-Np required to elicit half maximal response (EC_{50}; dotted lines) is given (n = 5–9 per condition and genotype; p = 0.018). The values are mean ± SEM.

(E) Immunodetection of PTCH1 and GLI2 in the CMZ of retinal explants treated with buffer or SAG. In control and \textit{Lrp2}^{-/-} tissues, PTCH1 is present in apical vesicles and at the cells surface (a and b) and enriched in GLI2+ cilia upon SAG stimulation (arrowheads in c and d). No colocalization is seen for LRP2 (green) and PTCH1 (red) in buffer (e) or SAG treated (g) CMZ explants. For comparison, the inset in (e) depicts colocalization of LRP2 and PTCH1 in the E10.5 RDVM. The scale bar represents 5 \( \mu \text{m} \).

(F) Explants of E16.5 CMZ or E8.5 RDVM were incubated with GST or GST-SHH-N for 2 hr and immunostained for PTCH1 (red) and ligand (green). The efficient uptake of GST-SHH-N is seen in control CMZ (c) and RDVM (g), but not in \textit{Lrp2}^{-/-} deficient tissues (d and h). No uptake of GST is seen in any condition (a, b, e, and f). The \textit{Lrp2}-mediated uptake of GST-SHH-N results in cointernalization of PTCH1 in the RDVM (yellow signals in g), but fails to cointernalize PTCH1 in the CMZ (green signals in c). The scale bar represents 10 \( \mu \text{m} \) (a) or 50 \( \mu \text{m} \) (b).

(G) Modulation of SHH signaling by LRP2. Under basal conditions (a), binding of SHH to PTCH1 results in internalization of PTCH1 and in pathway activation. In the RDVM (b), \textit{Lrp2} promotes signaling by interacting with PTCH1 to facilitate high-affinity binding and internalization of PTCH1/SHH complexes. The SHH is recycled via Rab11+ sorting pathways. In the CMZ (c), \textit{Lrp2} fails to interact with PTCH1 and directs lysosomal catabolism of the internalized morphogen (c). See also Figure S7.
to SHH, we scored the response of control and mutant explants to a range of SHH concentrations (determined by semiquantitative western blotting; Figure S7A). When the dose-response curves were determined, the effective concentration of SHH-Np to elicit a half-maximal proliferative response (EC_{50}) was 0.06 μg/ml in controls, but 0.02 μg/ml in the Lrp2 null CMZ (Figure 7D; p = 0.018). Thus, LRP2 activity in the control CMZ increased the threshold required to elicit a mitogenic response.

In the RDVM, LRP2-mediated internalization of SHH results in concurrent internalization of PTCH1, a prerequisite for pathway activation (Christ et al., 2012). Accordingly, we explored the cellular fate of PTCH1 in the CMZ in the presence or absence of SHH ligand. The specificity of the PTCH1 antiserum was confirmed on E16.5 eyes from mice with conditional inactivation of SHH ligand. The specificity of the PTCH1 antiserum was confirmed on E16.5 eyes from mice with conditional inactivation of SHH ligand. (Christ et al., 2012). The specificity of the PTCH1 antiserum was confirmed on E16.5 eyes from mice with conditional inactivation of SHH ligand. (Christ et al., 2012). In line with the lack of colocalization, LRP2-dependent internalization of GST-SHH-N in the control CMZ failed to efficiently cointernalize PTCH1 with the ligand (Figure 7F, c), whereas cointernalization of PTCH1 and GST-SHH-N was obvious in the RDVM (Figure 7F, g). These findings suggested distinct cellular mechanisms in the CMZ and RDVM that distinguish the ability of LRP2 to functionally interact with PTCH1 and to direct SHH to lysosomal versus recycling fates.

In conclusion, our data substantiated a role for LRP2 in control of SHH signaling that extends forebrain formation. In the retina, the endocytic activity of this receptor limits the distal spread of the SHH activity domain and protects RPC in the CMZ from mitogenic stimuli, a process that otherwise results in hyperplasia of the CB and in hereditary buphthalmos.

**DISCUSSION**

**LRP2 Deficiency Causes Ectopic Induction of SHH Activity in the Retinal Margin**

SHH is essential for several steps of mammalian eye development. Initially, SHH secreted from the prechordal plate induces formation of the optic primordia from a single eye field (Zhao et al., 2012). At subsequent stages of eye development, SHH produced locally by retinal ganglion cells assumes an important function in patterning of the neural retina by acting on RPC (Wang et al., 2005). In LRP2-deficient mice, formation of the optic pits proceeds normally as judged by the presence of two laterally placed eyes (Figures 1C and 1D). Proper establishment of bilateral eye structures is likely due to the fact that LRP2 deficiency does not impair proliferation of SHH in the prechordal plate (Christ et al., 2012).

While LRP2 deficiency does not impair SHH-dependent patterning of the optic pits, it affects local SHH activity during later stages of retina development. The source and the activity domain of SHH in the neural retina are not altered by LRP2 deficiency, as Shh and markers of postmitotic retinal cell types are properly expressed (Figure 2). Also, the postnatal retina shows correct stratification (Figures 1H and S1E). However, LRP2 deficiency causes a shift of the peripheral boundary of the SHH activity domain into the distal retinal margin. This fact was documented by increased signals for retinal ganglion cell marker Brn-3b (Figures 2U, 2V, and S2E), as well as for progenitor cell markers Sox2 and Vsx2 (Figures 4A–4C) in the mutant CMZ. Enhanced SHH activity is the likely cause of this phenotype, as increased levels of downstream targets Gli1, Hes1, and Ptc1 (Figures 3B–3D), and of Gli1-lacZ activity (Figure 3E) are seen in this tissue. As a consequence of increased SHH signal, the RPC pool in the mutant CMZ is expanded and the proliferative capacity of the retinal margin enhanced (Figure 4). Expression levels for Zic2 and Isl1, marking ipsilateral and contralateral projecting ganglion cells, respectively, are not significantly altered in the Lrp2^{-/-} eye (Figure S2E). A decrease in ipsilateral projections had been documented in mice lacking Boc before (Sanchez-Arrones et al., 2013). Thus, LRP2 activity mainly impacts the size of the progenitor cell pool in the CMZ, while differentiation to more specialized retinal cell types may require additional regulatory signals. LRP2-dependent expansion of the RPC pool may occur through SHH-induced proliferation of multipotent progenitor cells as suggested by double labeling of cells for BrdU and Sox2 (Figure S4E). Alternatively, CMZ cell types may adopt a partial RPC fate in response to SHH.

A function for LRP2 in the retinal margin is supported by its predominant expression in the CMZ (Figure S2). The SHH pathway is the target of receptor (dy)fuction, as other morphogen pathways are not altered (e.g., Ihh and BMP4) (Figures 3A and S3B) or decreased secondarily as shown by down-regulation of WNT activity by SAG (Figure 5D). Enhanced RPC proliferation as a consequence of increased Hh signaling is also seen in mice heterozygous for the Ptc1 gene defect (Moshiri and Reh, 2004) or in animals lacking Sufu, an inhibitor of the SHH pathway (Cwinn et al., 2011). In contrast, a WNT signaling defect in mice lacking β-catenin in the retina causes a decrease, rather than an increase, in the progenitor cell pool (Liu et al., 2007). Also, blockade of WNT activity by JW55 in this study failed to induce cell proliferation in the CMZ (Figure 5E). Consistent with the main role of SHH to stimulate proliferation (Sakagami et al., 2009; Wang et al., 2005), ectopic SHH activity in the Lrp2^{-/-} CMZ enhances proliferative capacity, impairing the quiescent character of RPCs in the CMZ (Figure 4).

**LRP2 Defines the Boundary of the SHH Activity Domain in the Retina**

All components of the SHH signaling machinery, including primary cilia, SMO, PTC1, and GLI2 are present in the wild-type CMZ as documented by ISH, immunohistochemistry, and qPCR. Also, the wild-type CMZ avidly reacts to SHH-Np (Figures 7A–7D and S7B) and SAG (Figure 5C) with proliferative responses equaling that of Lrp2^{-/-} tissue. The fact that SMO is excluded from primary cilia in the control CMZ indicates lack of sufficient SHH signal in the retinal margin of the wild-type eye. This hypothesis was confirmed by quantitative description of the response of the CMZ to exogenous SHH-Np. In control tissue, the EC_{50} is increased 3-fold, but the maximum level of response
unchanged as compared to the \( Lrp2^{+/−} \) CMZ (Figure 7D). This kinetic is representative of a competitive inhibitor (LRP2) present in the control, but absent from the mutant condition.

Because LRP2 delivers SHH to lysosomes in CMZ cells (Figures 6F and S6C–S6E) and antagonizes SHH signaling in trans in luciferase reporter assays (Figure S6I), we propose a model whereby the endocytic activity of this receptor provides a morphogen sink that depletes the ligand from the peripheral margin. Most importantly, this model is supported by the accumulation of endogenous SHH protein in the mutant CMZ (Figure 6D). Accumulation of SHH in the CMZ is not caused by increased expression, as levels of SHH in the neural retina or levels of \( Shh \) transcripts in the neural retina or CMZ are not increased in the \( Lrp2^{−/−} \) compared to control eyes. In non-mammalian systems, the ability of Hh binding proteins to contain the spread and activity of this morphogen has been shown in the wing disc (Bilioni et al., 2013; Yan et al., 2010) and ovaries (Hartman et al., 2010) of the fruit fly or in the optic vesicle of zebrafish and chick embryos (Cardozo et al., 2014).

**Loss of Quiescence Results in Hyperproliferation of the Retinal Margin and in Eye Pathology**

Aberrant proliferation of the CMZ in Lrp2 mutant mice proceeds into the postnatal period (Figures S4C and S4D) and coincides with hyperplasia of the CB in newborn mice (Figure 1I). The increase in postnatal eye size in \( Lrp2^{−/−} \) animals is most likely caused by hyperproliferation of the embryonic CMZ, as mice with conditional \( Lrp2 \) inactivation in the adult lack eye pathology (Storm et al., 2014). High myopia (nearsightedness), a defect caused by increased axial length, is also a consistent feature seen in individuals with Donnai-Barrow syndrome (Pober et al., 2008). While intraocular pressure cannot be determined accurately in neonatal mice, glaucomatous changes, such as thinning of the retina and loss of retinal ganglion cells, argue for an increased pressure as the cause of the buphthalmos in \( Lrp2^{null} \) animals. This conclusion is supported by the identification of mutations in \( lp2 \) in zebrafish in ENU screens for glaucoma genes (Veth et al., 2011). Possibly, quiescence of the mammalian CMZ assures integrity of the CB that produces the vitreous and causes glaucoma when dysfunctional.

**Context-Dependent Function of LRP2 in the SHH Pathway**

The ability of LRP2 to antagonize SHH signaling in the CMZ is surprising, considering its role as an activator of this pathway in the RDVM (Christ et al., 2012). What may be the molecular concept that determines context-dependent stimulation or repression of the SHH pathway by this receptor? Based on analysis of endogenous SHH pathway components, two concurrent mechanisms seem plausible, namely, the cell-type specific control of trafficking of SHH and of PTCH1 by LRP2 (Figure 7G). In the RDVM, LRP2 closely colocalizes with PTCH1 on the apical cell surface. Binding of SHH to LRP2 results in efficient internalization of ligand and PTCH1, and in subsequent pathway activation. SHH internalized by LRP2 is delivered to recycling endosomes, possibly destined for re-secretion to increase local morphogen concentration in the target field (Christ et al., 2012). In the CMZ, SHH internalized by LRP2 awaits a different fate as it is directed to lysosomes, depleting the ligand from the retinal margin. Also, LRP2 fails to induce cointernalization of PTCH1 (Figure 7F), preventing recruitment of SMO into cilia (Figure 6B). A role for LRP2 as SHH clearance receptor is supported by the accumulation of endogenous SHH in the mutant CMZ (Figure 6D). Obviously, binding of SHH to PTCH1 in the mutant CMZ results in cellular uptake of morphogen and receptor as judged from ectopic pathway activation in this tissue. However, the low levels and the relatively poor clearance capacity of PTCH1 as compared to the highly efficient endocytic receptor LRP2 likely precludes intracellular accumulation of endogenous SHH or exogenously added GST-SHH-N in the mutant CMZ to levels detectable by immunohistology. Also, much of the SHH ligand building up in the mutant CMZ may be dispersed in the intercellular space due to lack of a clearance pathway, further impeding immunodetection.

The capability of LRP2 to act in a context-dependent manner is shown by its association with distinct cytosolic adaptors that determine routing of the receptor through cell type specific sorting pathways (Shah et al., 2013). Concerning control of LRP2 and PTCH1 interaction, a similar mechanism is operable in the WNT pathway with transmembrane proteins Kremen 1 and 2 blocking the ability of LRPS/6 to functionally interact with the WNT receptor frizzled (Mao et al., 2002). Further identification of factors that govern the cell-type specific interaction of LRP2 with SHH and PTCH1 will shed light on an important molecular concept whereby this SHH binding protein assumes functions as activator or inhibitor to fine-tune the target response to SHH.

**EXPERIMENTAL PROCEDURES**

**Mouse Models**

Mice carrying \( Lrp2 \) null alleles due to targeted (Spoelgen et al., 2005) or ENU-induced (Zarbalis et al., 2004) gene disruption have been described before. Where applicable, these lines were crossed with the \(Gil1\_LacZ\) (JAX; Stock 008211) or the \(Tcf/Lef\_LacZ\) reporter lines (Mohamed et al., 2004). Experiments involving animals were performed according to institutional guidelines following approval by local authorities (X0917/12).

**Retinal Explant Cultures**

Retinal explants were isolated from E16.5 mouse eyes as published (Liu et al., 2007). The explants were cultured for 24–48 hr at 37°C, with 5% CO2 and 95% humidity. Where indicated, the culture medium (Wang et al., 2005) was supplemented with 1–5 µM KAAs-cyclopamine (Calbiochem), 800 nM SAG (Alexis Biochemicals), 5 µM tankyrase inhibitor JW55 (Calbiochem), or 0.05–0.20 µg/ml SHH-Np in conditioned medium from HEK293 cells stably secreting SHH-Np (SHHN-293 cells; provided by M. Kato, Stanford School of Medicine). For ligand uptake, retinal explants were incubated for 2 hr with 5 µg/ml GST-SHH-Np (produced in BL21 bacteria using vector pSh1-2tk) or with 10 µg/ml lactoglobulin (labeled with Alexa-488 using the Protein Labeling Kit; Life Technologies). Thereafter, explants were washed and fixed in 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature and processed for routine cryo-sectioning.

**BrdU Labeling In Utero and in Explants**

For BrdU incorporation experiments in retinal explants, the tissue was pulsed with 10 µM BrdU (Sigma) for 1 hr. To label proliferating cells in the CMZ of E16.5 embryos, BrdU was injected intraperitoneally into pregnant females at 50 mg/kg body mass and the animals sacrificed 1 hr later. For quantification of BrdU+ cells, cryo-sections of eyes or explants were subjected to standard immunohistology using rat anti-BrdU antibody (1:100; AbD Serotec), followed by biotin-streptavidin amplification procedure. The number of BrdU+ cells in the CMZ of retinal explants was counted on two sections through the medial plane of the lens per retinal explant for a total of 5–12 animals per genotype.
and condition. In embryonic eyes, the CMZ was defined as the region of interest and immunofluorescence intensities quantified using LAS AF software. Signal intensities were determined on 3–4 sections per eye for a total of 5–12 animals per genotype and condition.

Statistical Analysis
Statistical significance was calculated using GraphPad Prism 5/6 software. Results comparing two groups were analyzed either by Student’s t test (Figures 3D and 4C) or by Mann-Whitney U test (Figures 1G, 4B, 4E–4G, 6C, 5S1B, 5S2E, 5S3D, 5S4B, 5S4D, and 5S8). Results in which more than two groups and different treatments were compared to each other were analyzed by two-way ANOVA followed by Bonferroni post test (Figures 5B, 5C, and 7C). Results shown in Figure 6D were analyzed using one sample t test. Data in Figure 7D were analyzed using a sigmoidal dose-response (variable slope) equation. Values are indicated in the figure legends.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.develcel.2015.09.001.

AUTHOR CONTRIBUTIONS
A.C., A. Christa, J.K., and S.B. performed experiments and evaluated data. A. Christ, J.C.E., V.A.W., A.H., and T.E.W. conceived experiments and evaluated data. T.E.W. wrote the manuscript.

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